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(64) A method of determining dTk isoenzyme activity and the use thereof.

(57) A method of determining dTk isoenzyme levels of a human or animal body fluid or cell sample is disclosed, which comprises the steps of reacting said sample with a substrate for said isoenzyme in the presence of a phosphate donor and a buffer system, and measuring the amount of phosphorylated product formed, said amount being proportional to said isoenzyme level. The method is characterized by using in combination

- a) a buffer system at pH ranging from 5 to 9 and being present in a concentration of no more than 250 mM,
- b) a substrate consisting of 2'-deoxy-5-halouridin, part of which has a radio-labelled 5-halogen, and being present in a concentration ranging from  $2 \times 10^{-9}$  -  $5 \times 10^{-6}$  M, and
- c) the phosphate donor being present in a concentration not exceeding 20 mM.

The sensitivity of the method permits measurement of minute amounts of dTk, and the method can be used for e.g. diagnosis, prognosis and monitoring of diseases involving altered ATP mediated dTk activity, such as cancers, tumours, and certain virus infections, or altered CTP mediated dTk activity, such as HSV type 1, type 2, and VZV infections.

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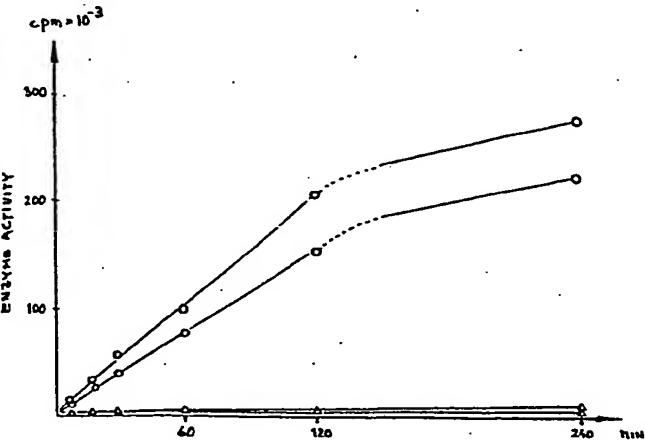


FIG. 1A

A method of determining dTk isoenzyme activity and the use thereof.

The present invention relates to a method of determining the levels of the isoenzyme dTk (deoxythymidine kinase) in human or animal body fluids or cell samples. The invention further relates to the use of said method for the diagnosis and prognostics of diseases dominated by ATP (adenosinetriphosphate) mediated dTk activity, such as cancers, tumours and certain viral infections as well as of diseases characterized by CTP (cytidinetriphosphate) mediated dTk activity, such as HSV (Herpes Simplex Virus) type 1 and type 2 and VZV (Varicella Zoster Virus) infections. The invention also relates to the use of said method for dTk isoenzyme typing.

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Background of the Invention

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The enzyme deoxythymidine kinase (dTk) provides the eucaryotic cell with a means for utilizing deoxythymidine (dT), which is not an intermediate in the thymidylate synthesis de novo. For this reason dTk is considered a salvage enzyme, introducing dT into the DNA metabolism. As the major dTk form of mammalian cell is only present during cell division, the dTk has been denominated scavenger enzyme.

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Three different cellular isoenzymes in human cells have been described. The cytosolic dTk, called dTk-F, which occurs in optimal amounts in dividing cells (stages G1 to S) (Bello, Exptl. Cell. Res. 89:263, 1974; Littlefield, Biochim. Biophys. Acta. 115:398, 1966) and is more or less absent in resting cells. In humans this enzyme is coded for in chromosome 17 near the galactokinase locus. The second cellular isoenzyme is the mitochondrial, denominated dTk-A, which is present in the mitochondrial matrix. The activity of this dTk remains relatively constant during the different cell stages (Adelstein et al; Develop. Biol. 26:537, 1971), and dTk-A is coded for by chromosome 16. The third dTk, a minor activity called dTk-B, has only been reported in continuous cell lines HeLa and KB and is said to be confined to the inside of the mitochondrial membrane (reviewed by Kit, Pharmacol. Ther. 4:501, 1979).

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The three cellular dTks differ in biochemical properties. The dTk-F and dTk-B, which are quite similar, are distinguished, besides as to localization, by isoelectric focusing, having different pI, and by electrophoretic mobility. In contrast to dTk-F and dTk-B, the dTk-A accepts cytidinetriphosphate (CTP) as a phosphate donor and is not as sensitive as the others to dTTP (deoxythymidine triphosphate) feedback inhibition. The dTk-A also phosphorylates deoxycytidine (dC) and is inhibited by dCTP (reviewed by Kit, Pharmacol. Ther. 4:501, 1979).

With regard to viruses, specific isoenzymes, coded by the viral genome, have been shown in the cell after infection with viruses from the Herpes group and the Pox group. Enzymatically the human virus specific dTks resemble

dTk-A, except for the vaccinia dTk which cannot utilize CTP as phosphate donor and not either can phosphorylate deoxycytidine. This dTk is easily distinguished from the human cellular dTks by electrophoresis (Kit et al., Progr. Med. Virol. 21:13, Karger Basel 1975). Both the HSV dTks and the VZV dTk have a broader spectrum of possible phosphate donors and accept different pyrimidines and pyrimidine analogues as substrates (Cheng et al, Biochim. Biophys. Acta. 452:370, 1976, and J. Virol. 31:172, 1979). The competitive blocking of dTk isoenzyme mediated dT conversion to dTmp (deoxythymidinemonophosphate) exerted by the dT-analogue 2'-deoxy-5-iodouridine (IUdR), has been known for a long time. The use of radio-labeled IUdR directly as a substrate to gain high sensitivity in assays of viral dTks was shown by us (Gronowitz & Källander, Infec. Immun. 29:425, 1980).

As mentioned dTk-F occurrence in cells is coupled to cell proliferation, and it is more or less absent in the differentiated cell (Munch-Petersen & Tyrsted, Biochim. Biophys. 478:364, 1977). Studies of dTk activity in transplantable mouse tumours have revealed high dTk activities with correlation to growth rate (Bresnick et al, Cancer Res. 29: 1969, and Cancer Res. 31:743, 1971). Recent reports have demonstrated enhanced dTk-F in peripheral blood lymphocytes of some patients suffering from malignant non-Hodkin's lymphoma and lymphatic leukemia (Ellims et al, Cancer Res. 41:691 and Brit. J. Haematol. 49:479, 1981). These researchers have also shown enhanced serum dTk levels in some patients from the non-Hodkin's group (Ellims et al, Blood 58:926, 1981). Due to the conventional dTk assay used, employing  $^3\text{H}$ -dT in high concentration ( $5 \times 10^{-6}\text{ M}$ ), dTk activity could only be found in a few patients with advanced disease. Further the dTk activity could be evaluated as a prognostic marker only if differential analysis regarding dTk-F and dTk-A was performed.

Conventionally dTk activity has thus been measured by following the conversion of either  $^3\text{H}$ - or  $^{14}\text{C}$ -labeled dT to dTmp with ATP as the phosphate donor. We have recently designed an improved dTk assay system for viral dTk isoenzymes, which uses  $^{125}\text{I}$ -Iododeoxyuridine (IUdR) as the substrate. The improved sensitivity obtained allowed the detection of dTk from as little as 25 HSV-infected cells. In contrast the conventional assay based on the use of  $^3\text{H}$ -dT, at a normally used concentration of  $10^{-5}\text{ M}$ , would require at least 450 times more enzyme. However, even though being useful in studies of viral dTks this improved assay system was not appropriate for long time assays of cellular dTk-F, partly due to the instability of this enzyme. The inability of this method of detecting the presence of minute dTk amounts in serum is easily extracted from the studies over occurrence of virus dTk blocking antibodies (dTk-ab). In these

studies more than 275 human sera were assayed for dTk-ab but disturbing serum dTk activities were found in only two cases (Gronowitz & Källander, Infec. Immun. 29:425, 1980; Gronowitz & Källander, J. Med. Virol. 8:177, 1981; Källander et al, Infec. Immun. 36:30, 1982).

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#### Summary of the Invention

It is an object of the present invention to provide a means for the diagnosis of certain virus infections and malignant tumours, including the monitoring of the malignancies for different purposes.

A further object of the invention is to provide a sensitive assay system  
10 capable of detecting minute amounts of dTk present in human and animal body fluids and cell samples, e.g. in serum, blister secretes, spinal fluid, vesicle fluid, and similar clinical specimens, which system is also designed to minimize the contribution of cellular dTk-A.

A further object of the invention is to provide a method of assaying for  
15 dTk-F for use in the detection and prognostics of tumour diseases, and for the monitoring of alterations in the disease, and detection of relapses.

A still further object of the invention is the use of said assay method in the evaluation of metastases in connection with lung cancer of the oat cell type.

Still another object of the invention is the use of said assay method in  
20 connection with leukemia for determining, by measurement of dTk present in spinal fluid, if metastases are present in the cerebro-spinal system.

Another object of the invention is the use of said assay method as a group specific marker for certain infections in connection with viral diseases.

A further object of the invention is the use of a modification of said  
25 assay method for achieving specific detection of herpes virus specific dTks present in e.g. serum or vesicle fluid.

The above and other objects of the invention will be explained in detail in the following description of the assay method and its applications according to the invention.

30 The invention thus provides an improved assay system for the measurement of dTk levels, especially dTk-F levels in e.g. serum. This assay system is considerably more sensitive than prior art systems, and it has been found that the sensitivity is so high, that it is possible to use the assay system, which is very simple to practice, not only for determining pathological dTk levels, but even normal levels in healthy individuals (compare Figures 1 and 6).  
35 This assay system was not only found to be very sensible and reproducible for dTk-F determinations, giving linear turnovers for more than two hours, but was also found to give 2-6 times higher turnovers than prior art systems with the

viral dTks. Another important advantage, compared to the conventional assay system, is the comparatively low turnover of dTk-A, this isoenzyme giving minimal contribution when measuring dTk-F or viral dTk in non-purified samples.

5 A variant of this assay system, using CTP instead of ATP as the phosphate donor, was found to be useful for specific detection of herpes virus induced dTk when samples to be analysed were preincubated with anti-herpes viruses dTk blocking sera.

10 The advantageous and unexpected technical effects described herein are according to the invention obtained by the use of a combination of a buffer system of low concentration and ionic strength, a specific type of radio-labelled substrate, which is present in low concentration, and a low concentration of a phosphate donor.

15 In its broadest aspect the invention relates to a method of determining dTk isoenzyme levels in a human or animal body fluid or cell sample wherein said sample is reacted with a substrate for said isoenzyme in the presence of a phosphate donor and a buffer system, and wherein the amount of phosphorylated product formed is measured, said amount being proportional to said isoenzyme level. According to the invention said method is characterized by using, in  
20 combination

- a) a buffer system at pH ranging from 5 to 9 and being present in a concentration of no more than 250 mM,
- b) a substrate consisting of 2'-deoxy-5-halouridin, part of which has a radiolabelled 5-halogen, and being present in a concentration ranging from  $2 \times 10^{-9}$  -  $5 \times 10^{-6}$  M, and
- c) the phosphate donor being present in a concentration not exceeding 20 mM.

25 In contrast to the earlier described assay system using  $^{125}\text{I}$ -IUDR as substrate, the assay system according to the invention gives a linear turnover of substrate for the cellular dTk-F for more than two hours, and the detection sensitivity was drastically increased, as can be seen in Figure 1. This Figure is a comparison between the assay system according to the invention (-o-o-o-) and said prior art assay system (-Δ-Δ-Δ-) (Gronowitz and Källander, Infec. Immun. 29:425, 1980). A = two sera containing human dTk-F originating from patients with leukemia. B = HSV type 2 dTk of cell culture origin. Total available  $^{125}\text{I}$ -IUDR was  $550 \times 10^3$  cpm.

Brief Description of the Drawings

Fig. 1A and B are diagrams showing enzyme activity plotted against time and illustrating the drastically improved sensitivity of the claimed method compared to the closest prior art.

5 Figs. 2A and B illustrate - by way of tlc graphs - the reduction in radiochemical purity of the preferred substrate according to the invention,  $^{125}\text{I}$ -IUDR, on storage.

Fig. 3 is a diagram illustrating the calculation of enzyme activity in units when using an internal substrate control.

10 Fig. 4 is a diagram illustrating the inhibitory effect of high serum concentrations in dTk measurements.

Fig. 5 illustrates - by way of diagrams - the typing of viral dTk in different clinical specimens.

15 Fig. 6 is a diagram illustrating the normal distribution of dTk activity in blood donors and pregnant women.

Fig. 7 is a diagram illustrating dTk activities, measured according to the invention, in sera from patients with infectious mononucleosis and related to time after onset of illness.

20 Fig. 8 is a diagram illustrating dTk activities found in sera from patients suffering from various virus infections, mycoplasma pneumoniae, and psittacosis.

Figures 9a and 9b are diagrams illustrating the correlation between serum dTk activity, as measured according to the invention, and the stage of the disease for patients suffering from NHL.

25 Figures 10a and 10b illustrate the probability of survival for NHL patients related to the pretreatment serum dTk levels as measured according to the invention.

Figures 11 and 12 are diagrams illustrating longitudinal studies of serum dTk levels for NHL patients with varying clinical course.

Description of Preferred Embodiments

30 In a preferred embodiment of the invention said buffer system is present in a concentration of less than about 150 mM, especially being about 100 mM. The pH of the buffer is preferably in the range 6.5-8, especially about 7.4. The best results have been obtained with buffers devoid of maleate, and  
35 lacking reactive or primary amino groups, in particular buffers substantially devoid of TRIS-maleate, HEPES buffer being the especially preferred buffer.

The substrate should be a radio-labelled thymidine analogue, especially a 2'-deoxy-5-halogen-uridine, wherein the label preferably is a reactive halogen isotop. The preferred substrate is  $^{125}\text{I}$ -2'-deoxy-5-iodouridine. The preferred

substrate concentration is in the range  $1 \times 10^{-8} - 5 \times 10^{-7}$  M, especially about  $10^{-7}$  M.

The preferred phosphate donor for dTk-F determination is ATP, and the preferred phosphate donor for viral dTk determination is CTP. The preferred concentration of the phosphate donor is less than about 10 mM, especially about 0.5 - 5 mM. Phosphate donors known per se to have the same isoenzyme specificity as ATP and CTP may also be used.

$Mg^{2+}$  should preferably be present in at least equimolar concentration relative to the phosphate donor. A reducing agent, such as dithiothreitol, is 10 preferably also present.

The measurement of the phosphorylated product can be made by procedures known per se (see e.g. Gronowitz et Källander, Infec. Immun. 29:425, 1980).

The application of the assay system according to the invention for 15 detection of dTk activities in clinical specimens, e.g. serum or blister secrete, provided a new tool for diagnosis of certain virus infections, and malignancies. Also, abnormal serum dTk levels (e.g. > 10 units), obtained with a single dTk assay with the procedure described, were found to be an excellent marker for prognostic use and so also sequential dTk values in monitoring the progression 20 or regression of malignant disease. Furthermore in these patients serum dTk levels were useful as a marker of relapses and in evaluation of therapy effects. All, as exemplified below.

In the preferred embodiment, which has been practiced when performing the tests reported below,  $^{125}I$ -IUDR is used as the substrate. The 25 composition and proportions, including final concentrations, of the preferred assay system are shown in Table 1 below.

TABLE I  
Composition of buffer used in dTk assay

Component	Assay mixture	Final concentration <sup>a</sup>
bHEPES	0.1 M	86 mM
MgCl <sub>2</sub>	17 mM	15 mM
KCl	20 mM	17 mM
NaF	1,2 mM	1,0 mM
ATP	4.6 mM	3.9 mM
Dithiothreitol	2.7 mM	2.3 mM
Bovine Albumine	0.33 mg/ml	0.28 mg/ml
Glycerol	6.6 %	5.7 %
IUDR	-	1.1 x 10 <sup>-7</sup> M
pH	7.4	7.4

<sup>a</sup>Proportions of one double sample is: 51.5 µl assay mixture, 6 µl of substrate solution (final concentration of <sup>125</sup>I-IUDR 1 x 10<sup>-7</sup> M, 130-160 Ci/mM), and 2.5 µl of enzyme solution e.g. serum (added when starting the reaction), giving a final volume of 60 µl.

<sup>b</sup>N-2-hydroxyethylpiperazine N-2-ethanesulfonic acid.

In general, one double sample is prepared, consisting of 51.5 µl reaction solution, which is mixed with 6 µl substrate solution immediately before use. The reaction is then started by addition of 2.5 µl enzyme solution (e.g. serum sample). From this standard volume of a total of 60 µl, two samples of each 25 µl are taken. The composition of assay mixtures for experiments consisting of several double samples, taken after different incubation times, are calculated by multiplying the volumes of the components in the standard solution with the desired number of double samples. The assay is performed at 37°C and all components are prewarmed 2 minutes before starting the reaction. The enzyme reaction is terminated by pipetting the sample onto a 1 cm<sup>2</sup> piece of Whatman DEAE-81 paper kept at 90-100°C. To separate the product from the substrate, the paper is washed four times in 6 mM ammoniumformate solution, once in distilled water and finally in methanol. The washings are performed in 1 liter glass vessels kept on magnetic stirring. 10-15 paper discs, kept in a colander, are processed simultaneously. The colander is moved to a new washing bath every five minutes. Finally the paper discs are counted in an automatized gamma counter.

In order to eliminate day-to-day variation in the dTk assay, the reaction velocities obtained were recalculated to units. The reason for utilizing units in defining enzyme quantitites is the variation in initial radiochemical purity, combined with the found biological decay of commercially available  $^{125}\text{I}$ -IUDR, which makes total radioactivity added per sample non-relevant for enzyme activity calculations. See Fig. 2, which shows separation of batches of commercially obtained  $^{125}\text{I}$ -IUDR on thin layer chromatography using precoated silica gel plates (Merck 60 F<sub>254</sub>). 15% methanol, 85% chloroform was used as eluent. A = profiles at day of delivery, indicating 62% radiochemical purity. B = the same batch after 150 days in refrigerator. Initially > 90% radiochemical purity was claimed by the manufacturers. Instead, an internal biologic control, measuring accessible radioactivity, was included in each assay. This control was a 100-fold excess of an HSV type 2 dTk preparation, giving extensive substrate exhaustion. The amount of radioactivity incorporated in a 1 hour assay by this control was found to be about 85% of the values of intact  $^{125}\text{I}$ -IUDR, as calculated from thin layer chromatography. Considering the level (85%) found for the biological control, 1 unit of enzyme will be the enzyme amount converting  $4.3 \times 10^{-15}$  moles of substrate per hour (under the conditions described). This relationship between unit and approximative molar turnover was chosen, as 1 unit will practically be expressed as about 1000 cpm, with the amount of isotope normally used (cf. Table 1). Another advantage with this biological isotope control, as it is processed in the same manner as the test samples, is that possible variations in units due to variations in product recovery are eliminated. The procedure for the calculation of units is exemplified in Fig. 3. Values for both the sample (-o-o-) and the control (-Δ-Δ-) are shown. A is the value of the internal control in cpm, which is proportional to the total available radiolabeled substrate. B is the value of product formed by the investigated

sample expressed as cpm per hour. Units (U) =  $\frac{B \times (S) \times v}{A \times 5.3 \times 10^{-15}}$ , where (S) is the substrate concentration (M), and v is the sample volume (liters).

Determination of serum dTk activities. Quantification of serum dTk was done in microtiter plates. For every 20 serum samples, background controls and an internal substrate control (see above) were included, and the set was subsequently processed as a unit. The amounts of serum sample used were 5  $\mu\text{l}$  (or less) per 120  $\mu\text{l}$  final volume, as more serum might be inhibitory. See Fig. 4. dTk activities were determined by sampling at 120 minutes. Enzyme activity as plotted against serum concentration per 60  $\mu\text{l}$  sample. (o-o) serum from an individual suffering from NHL, ( $\Delta-\Delta$ ) serum from an individual suffering from

active CLL. This inhibitory effect is probably due to the presence of nucleotides or nucleosides, such as e.g. thymidine, thymidinetriphosphate, or catabolic enzymes present in some sera. Double samples were taken after 60 and 120 minutes. Medium turn-over, per hour and 25 µl sample, after correction for background, was calculated from each double sample separately. The average velocity value of the 60 and 120 minutes sampling was then determined, and used in further calculations (see above), if < 20% variation was found.

5           Determination of dTk activities present in spinal fluid. Essentially the same procedure as for serum was used. The specific activity of <sup>125</sup>I-IUdR was increased to around 320 Ci/mM. Spinal fluids may be concentrated before the 10 enzyme determinations.

15           Qualitative determination of dTk isoenzymes. Clinical specimens, e.g. vesicle fluids or serum, containing dTk activity were preincubated with isoenzyme specific dTk blocking antibody and residual enzyme activity was determined according to the procedure described above. ATP in the assay mixture was replaced by CTP, resulting in exclusion of dTk-F activity. The specimens to be investigated were serially twofold diluted (usually starting at 1:6) in reaction solution, and 15 µl was transferred from each dilution to four wells in a micro titer plate, in order to achieve four identical dilution sets. Prediluted VZV dTk-blocking serum (25 ml) was added to one well, a HSV type 1 dTk-blocking serum to a second well, a HSV type 2 dTk-blocking serum to a third well and to the last well a negative serum. The mixtures were incubated at 37°C for 90 min., allowing the enzyme-antibody reaction to occur. Then the residual enzyme activities were determined. The reaction was started by the addition of 20 µl of a solution composed of 6 µl substrate solution mixed with 14 µl reaction solution. For each dilution the values obtained in the presence of the different antisera were recalculated as the percentage of the values obtained with the negative serum. Examples of such residual enzyme activities plotted against the log<sub>2</sub> dilution of specimens are shown in Fig. 5, wherein the residual activity after incubation with anti-VZV dTk serum (Δ - Δ), anti-HSV type 1 dTk (serum (●-●), and anti-HSV type 2 serum (○-○) in relation to control incubation with negative serum is plotted against different dilutions of the following samples: A = vesicle fluid from an individual suffering from an HSV type 2 infection; B = vesicle fluid from an individual suffering from an VZV infection; C = vesicle fluid from an individual suffering from HSV type 1 infection; D = serum from an individual suffering from VZV infection. From the inhibition patterns the dTk type can be easily established. (See also Table 2 below).

Table 2. Detection and typing of viral dTk activity in clinical specimens.

Sample type Vesicle fluid	Sample code	Enzyme activity <sup>a</sup> (cpm x 10 <sup>-3</sup> )	% Residual enzyme activity after incubation with sera			Immunological type of dTk	Clinical diagnosis
			VZV	HSV 1	HSV 2		
1		2.5 x 10 <sup>3</sup>	17	92	123	VZV	Herpes zoster
2		8.1 x 10 <sup>2</sup>	7	118	85	VZV	Herpes zoster
3		6.6 x 10 <sup>2</sup>	6	119	104	VZV	Varicellae
4		-	-	-	-	-	vesicles non viral origin
5		7.6 x 10 <sup>1</sup>	99	89	5	HSV 2	Genital Herpes
6		5.9 x 10 <sup>1</sup>	9	127	109	VZV	Varicellae
7		8.8 x 10 <sup>1</sup>	101	70	8	HSV 2	Genital Herpes
8		1.2 x 10 <sup>3</sup>	11	124	104	VZV	Herpes zoster
9		2.3 x 10 <sup>2</sup>	107	4	95	HSV 1	Herpes stomatitis
10		1.2 x 10 <sup>1</sup>	16	89	118	VZV	
11		2.8 x 10 <sup>3</sup>	5	87	91	VZV	Varicellae
12		6.5 x 10 <sup>2</sup>	7	109	116	VZV	Varicellae
13		1.5 x 10 <sup>2</sup>	10	92	89	VZV	Generalized Herpes zoster
serum samples							
A		2.8 x 10 <sup>1</sup>	18			VZV	Varicellae
B		1.2 x 10 <sup>2</sup>	18			VZV	Varicellae
C		1.6 x 10 <sup>2</sup>	8			VZV	Varicellae
D		3.4 x 10 <sup>1</sup>	12	96	85	VZV	Varicellae
E		4.0 x 10 <sup>1</sup>	16	114	85	VZV	Varicellae
F		9.6 x 10 <sup>1</sup>	11			VZV	Varicellae
control dTk from dTkA cultures							
VZV		1.0 x 10 <sup>2</sup>	120	111	98		
HSV 1		8.5 x 10 <sup>1</sup>	7	106	112		
HSV 2		1.3 x 10 <sup>2</sup>	96	11	94		
		7.5 x 10 <sup>1</sup>	112	89	9		

<sup>a</sup>Activity of control (sample incubated with negative serum) when using 5 µl sample and IUdR at a specific activity of 370 Ci/mole.

One important feature of the invention is thus the drastically increased detection sensitivity, which allows measurement even of normal serum levels of dTk-F in healthy individuals, as can be seen from Fig. 6, which shows the distribution of dTK activity found in sera from 99 blood donors (unfilled columns) and 25 pregnant women in the first trimester (shaded columns). The average is 2.4 units/ $\mu$ l with a standard deviation of 1.25.

Investigations of serum samples from individuals suffering from infectious diseases lead to the discovery of 10-40 times elevated dTK levels in serum from patients in acute stages of certain virus infections, e.g. mono-  
nucleosis infectiosa, rubella- and morbillivirus infections. Fig. 7 shows the dTk activity/ $\mu$ l formed in sera from patients with infectious mononucleosis as related to time after onset of illness. Symbols labeled with the same letter indicate sera from the same patient, and the dotted line shows the normal value for healthy individuals. Fig. 8 shows the dTk activity/ $\mu$ l found in acute (●) and convalescent (○) sera of patients suffering from different virus infections, mycoplasma pneumoniae, and psittacosis. The dotted line indicates a value, which is 4 times the normal value for healthy individuals.

Concerning herpes viruses the results indicate elevated serum dTk activities in connection with primary infections. No consequent increases in dTk activities (>10 units) were found in connection with other infections (Fig. 8). The characterization of the serum activities found has hitherto demonstrated the presence of Varicella Zoster Virus (VZV) specific dTk (Table 2) and cell dTk-F. It has also been demonstrated that the claimed method can be used for determining virus specific dTk present in vesicle fluids for rapid diagnosis of infections (Table 2). All serum dTk activities found in connection with viral infections disappeared gradually, reaching normal levels within 2-6 weeks.

Regarding patients suffering from malignant diseases, a limited study, using the assay method according to the invention, showed elevated serum dTk levels not only in patients suffering from malignancies originating from the lympho-proliferative system, but also in patients having malignancies of other cells with metastases in the lymphoproliferative system (see Table 3).

Extended studies have been performed with well defined large serum materials from patients suffering of lympho-proliferative diseases, such as non-Hodgkin's lymphoma (NHL), Hodgkin's disease (HD), cronic and activated lymphatic leukemia (CLL) and myelomas. Detailed results in the NHL group were as follows: normal serum dTk and enhanced dTk levels of different magnitude were found. These dTk levels correlated to staging of the disease, (see Fig. 9a which shows serum-dTk in 155 untreated patients with NHL

Table 3. dTk levels in sera from patients with different tumour diseases.

Type of disease	Status of disease	dTk activity (units/ $\mu$ l)
Lung cancer <sup>d)</sup>	no metastases	5
5 Lung cancer	no metastases	2
Lung cancer	metastases	163
Lung cancer	metastases	19
HD	metastases	108
10 HD	metastases	28
HD	no metastases	4
10 HD	no metastases	3
NHL	advanced stage	333
NHL	advanced stage	14
NHL	early stage	3
15 NHL	early stage	5
Adenocarcinoma	active	72
Leukemia	acute	40
Leukemia	acute	185
B-cell leukemia	active	52
20 CML	active	3500 a)
CML	active	45
CLL	active	62
CLL	active	18
25 CLL	semiactive	11
CLL	semiactive	12
CLL	in active	2.8 b)
Healthy individuals		2.4 c)
Leukemia acute	no metastase	0.07 e)
Leukemia acute	no metastase	0.20 e)
30 Leukemia acute	metastase	1.3 e)
Leukemia acute	metastase	1.6 e)

a) Therapy two days earlier.

b) Average value from 6 patients; values ranging between 0.5 and 5.4

c) See previous section.

35 d) All lung cancers in this study were of the oat cell type.

e) determination of dTk activity in spinal fluid, 5 $\mu$ l undiluted spinal fluid was used per 120 $\mu$ l final volume in assay.

Abbreviations used; HD; Hodgkins' disease

NHL; non-Hodgkin's lymphoma

CML; chronic myeloid leukemia

- CLL; chronic lymphatic leukemia

correlated to stage of the disease), i.e. the more advanced disease the higher the dTk value. When the dTk levels were related to the malignancy of the tumour cells (classification according to the Kiel system), then a good correlation between high dTk levels and malignancy was found (see Fig. 9b  
5 which shows serum dTk in 101 NHL patients in stages III-IV divided into three stages of malignancy)

The utility of the assay method according to the invention for prognostic purposes was demonstrated by highly significant differences in survival times between patient groups constructed by division according to  
10 measured dTk values < 10 units and > 10 units respectively. Fig. 10a shows the probability of survival for NHL patients in stages III-IV with pretreatment serum dTk < 10 units (-o-o-, n=50) and > 10 units (-●-●-, n=51). The numbers in parenthesis indicate remaining individuals at observation after 32 months. Fig. 10b shows  
15 the probability of survival for NHL patients in stages III-IV with "high grade" malignancy of tumour. Pretreatment level of serum dTk < 10 (-o-o-, n=11) and > 10 units (-●-●-, n=27). The numbers in parenthesis indicate remaining individuals at observation after 32 months.

Furthermore, longitudinal studies of serum dTk levels in NHL patients revealed alterations correlating to alterations of the disease, i.e. lower values  
20 upon regression of disease, higher upon progression and unaltered in constant disease. Fig. 11 shows the variations in the measured serum dTk levels for A three patients with progressive disease, B two patients treated to partial remission, and C four patients treated to complete remission. In Fig. 12 serum dTk is followed longitudinally in patients, where both progressive disease (a),  
25 and remission (b) occur. A) were two patients with progressive disease, who after change of therapy were treated to partial remission before the disease progressed again. B) were three patients treated to remission (A.H. and E.L. to partial, and G.E. to complete remission). All of them later relapsed. A.H. responded for a short time once more to therapy. These results clearly show  
30 the possibility to monitor the effect of therapy, and also to rapidly detect relapses both during and after treatment as increasing dTk values.

When using the assay method according to the invention in connection with the other above mentioned lympho-proliferative diseases variable serum dTk levels were found, e.g. in chronic CLL normal values were found, while half activated CLL gave low but pathological values (>10 units), and active CLL high. Possible uses of dTk levels, as exemplified for NHL, are also relevant for CLL. Moreover, studies of spinal fluid, derived from patients with leukemia, show presence of dTk when leukemic cells are present in the cerebro-spinal

system. Regarding cancer, the results found from patients with lung cancer of the oat cell type indicate the possibility to utilize serum dTk levels as a marker of metastases as well as for other cancers. These conclusions are evident from Table 3.

5       Naturally one has to be aware of possible transient elevated serum dTk level in patients with malignant disease, due to the above mentioned virus infections. However, these virus diseases are normally contracted in childhood, while malignant tumour diseases commonly is present in adults. Another factor relevant for the use of serum dTk levels for the described purposes, is the rare  
10      occurrence of elevated dTk activities indicated in a study of an unselected serum material, derived from patients with undefined disease, consecutively obtained for diagnostic purposes. Studies of medical records for these and other patients have shown that neither pathological values in liver function tests, nor leucocytosis are normally accompanied by enhanced serum dTK levels.

CLAIMS

1. A method of determining dTk isoenzyme levels of a human or animal body fluid or cell sample, comprising the steps of reacting said sample with a substrate for said isoenzyme in the presence of a phosphate donor and a buffer system, and measuring the amount of phosphorylated product formed, said amount being proportional to said isoenzyme level,  
5 characterized by using in combination
  - a) a buffer system at pH ranging from 5 to 9 and being present in a concentration of no more than 250 mM,
  - b) a substrate consisting of 2'-deoxy-5-halouridin, part of which has a  
10 radio-labelled 5-halogen, and being present in a concentration ranging from  $2 \times 10^{-9}$  -  $5 \times 10^{-6}$  M, and
  - c) the phosphate donor being present in a concentration not exceeding 20 mM.
2. A method according to claim 1, wherein said pH is from about 6.5-8,  
15 said substrate concentration is from  $10^{-8}$  -  $5 \times 10^{-7}$  M, and said phosphate donor concentration is from about 0.5-5 mM.
3. A method according to claim 1 or 2, wherein said buffer substance is essentially free from TRIS-maleate.
4. A method according to claim 1, 2 or 3, wherein said phosphate donor is  
20 selected from the group consisting of ATP and CTP.
5. A method according to claim 1, 2, 3 or 4, comprising the step of determining available radio-labelled substrate by including a control consisting of dTk isoenzyme in an excess sufficient to cause substrate exhaustion, said control being processed in the same manner as said sample before measuring the  
25 radio activity of said phosphorylated product.
6. Use of the method according to any one of claims 1 to 5 for the diagnosis of diseases involving altered ATP mediated dTk activity, such as cancers, tumours, and certain virus infections.
7. Use of the method according to any one of claims 1 to 5 for the  
30 diagnosis of diseases involving altered CTP mediated dTk activity such as HSV type 1, type 2, and VZV infections.

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8. Use of the method according to any one of claims 1 to 5 for dTk isoenzyme typing, comprising the step of preincubating said sample with dTk isoenzyme type specific blocking agents, such as antibodies.

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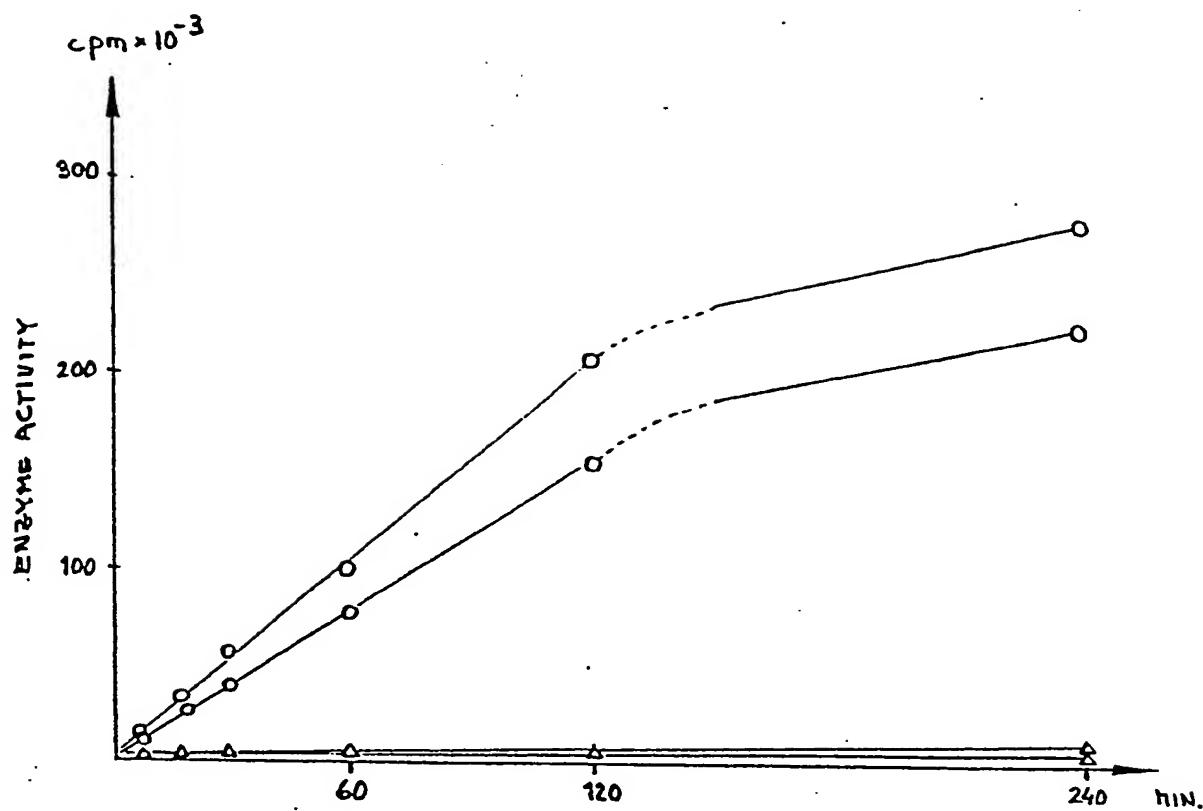


FIG 1A

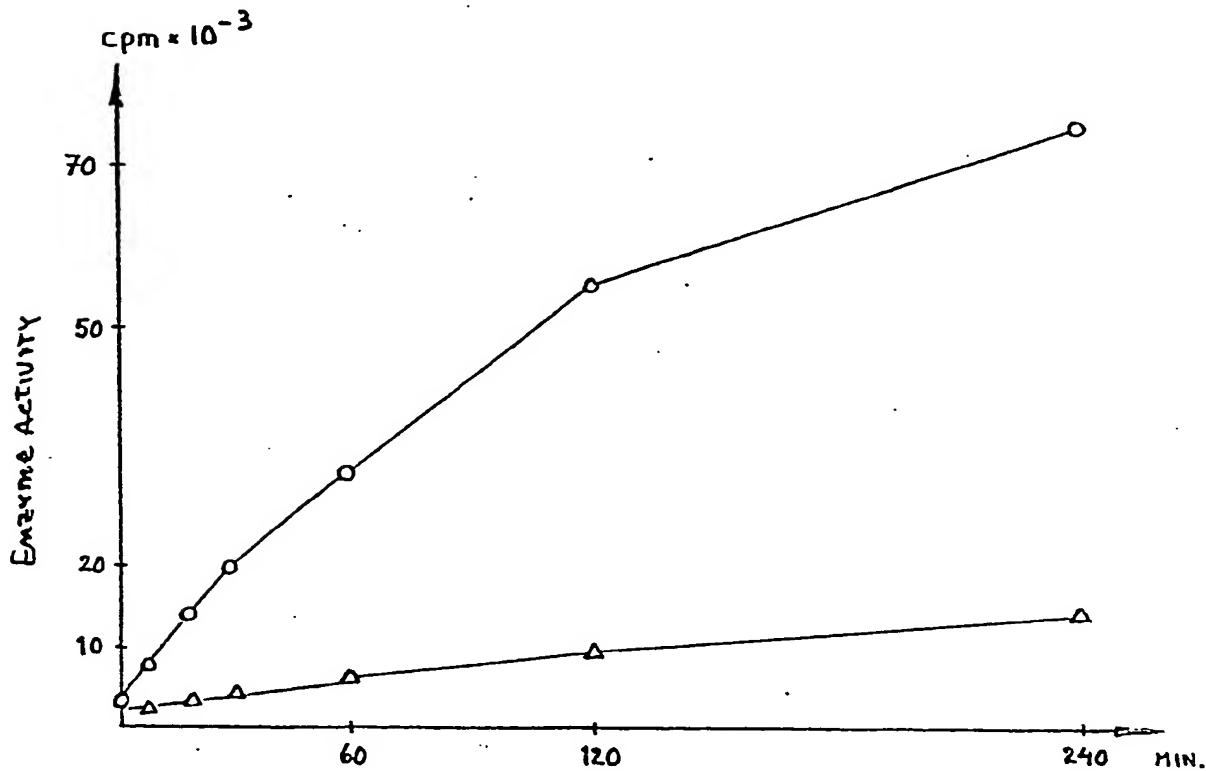


FIG 1B

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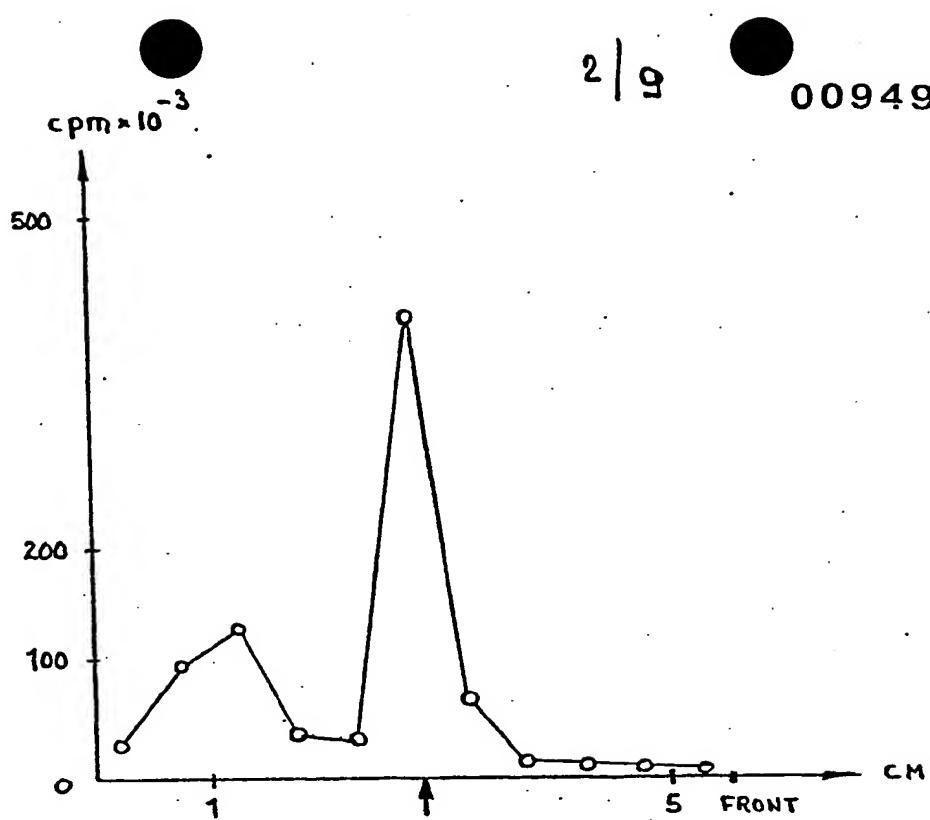


FIG 2A

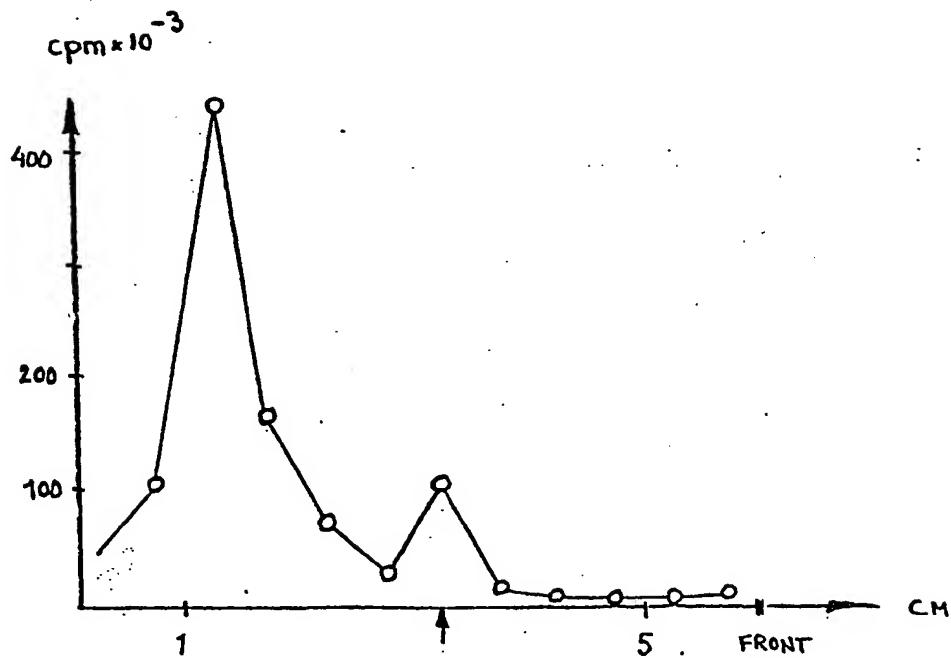


FIG 2B

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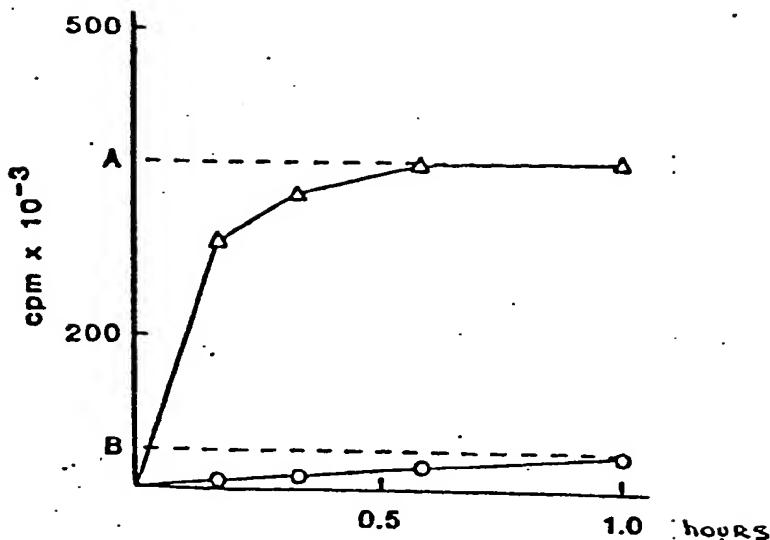


FIG 3

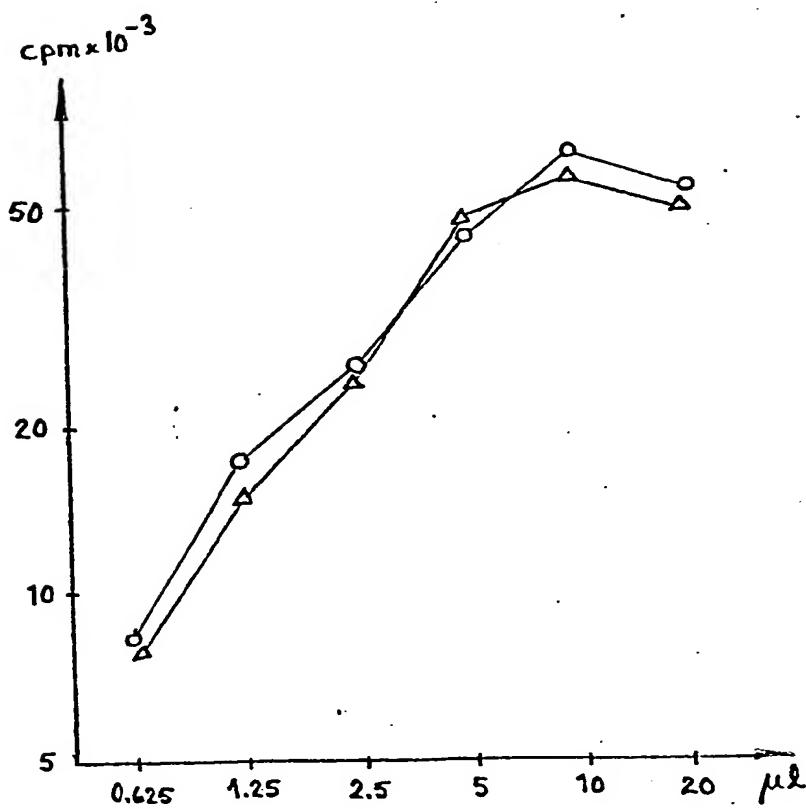
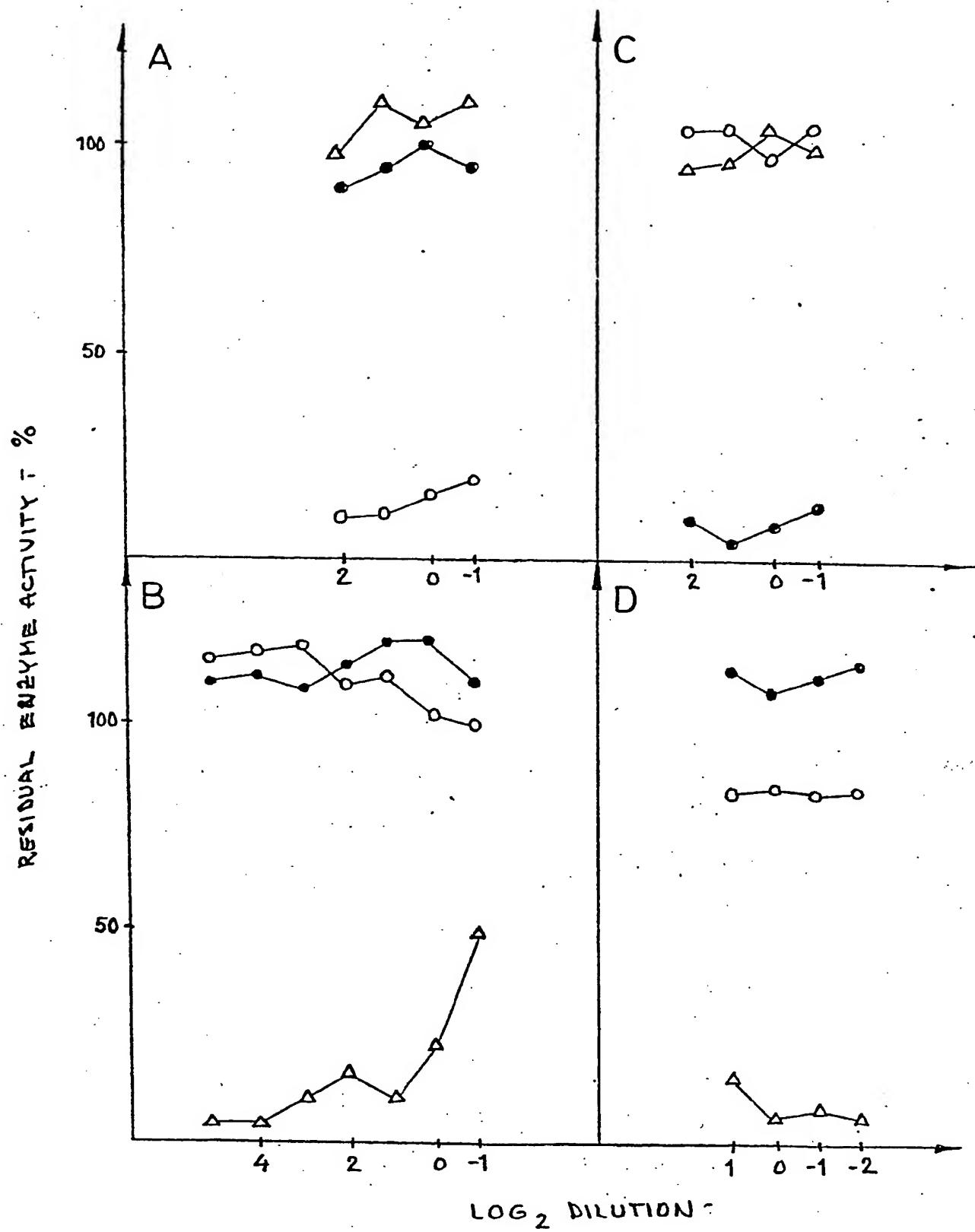


FIG 4

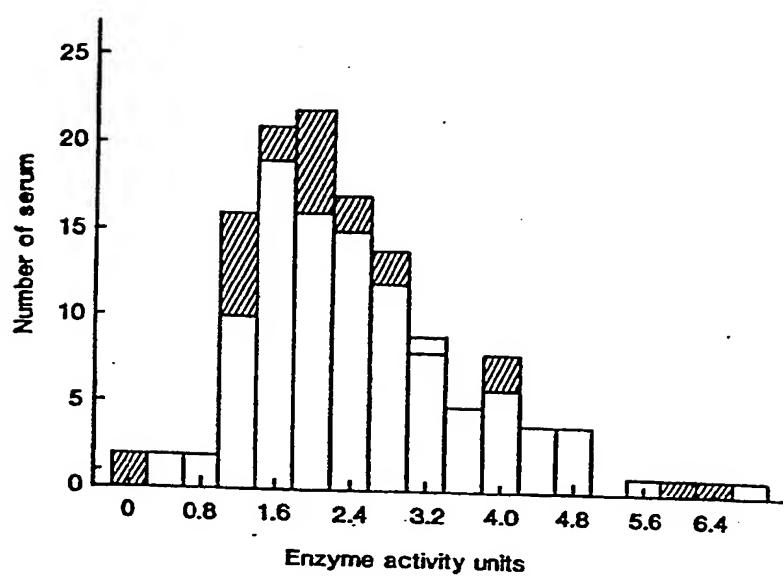
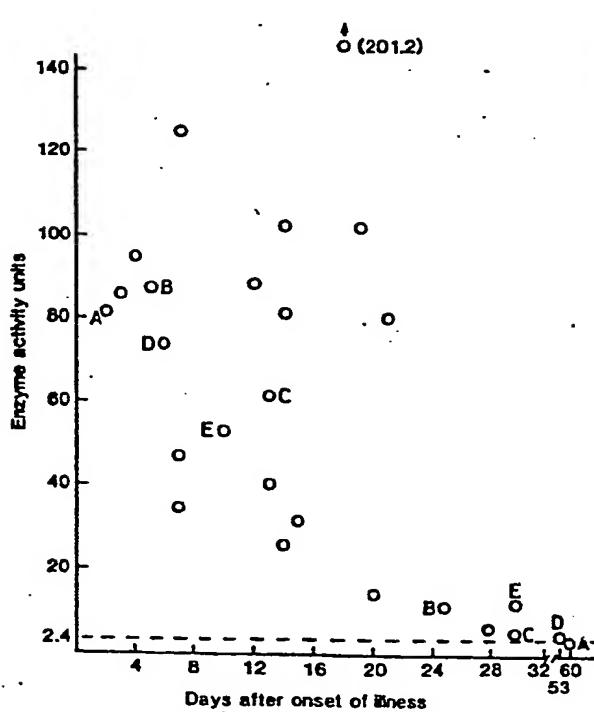
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FIG 5

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FIG. 6FIG. 7

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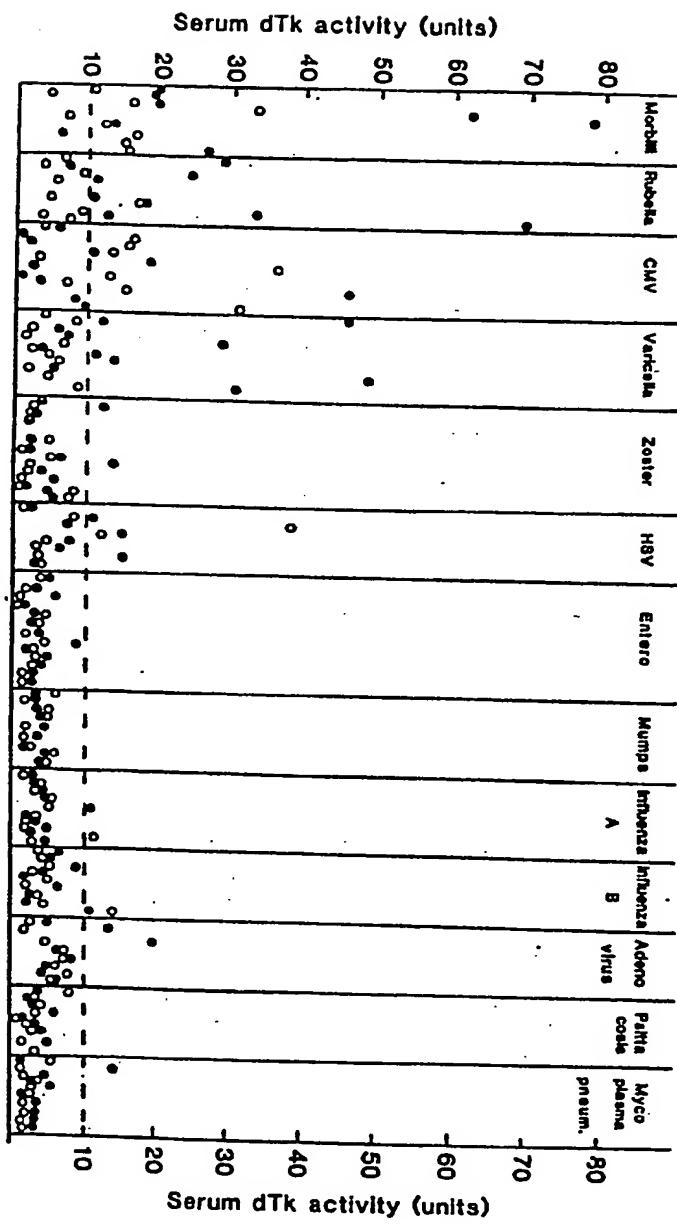


FIG. 8

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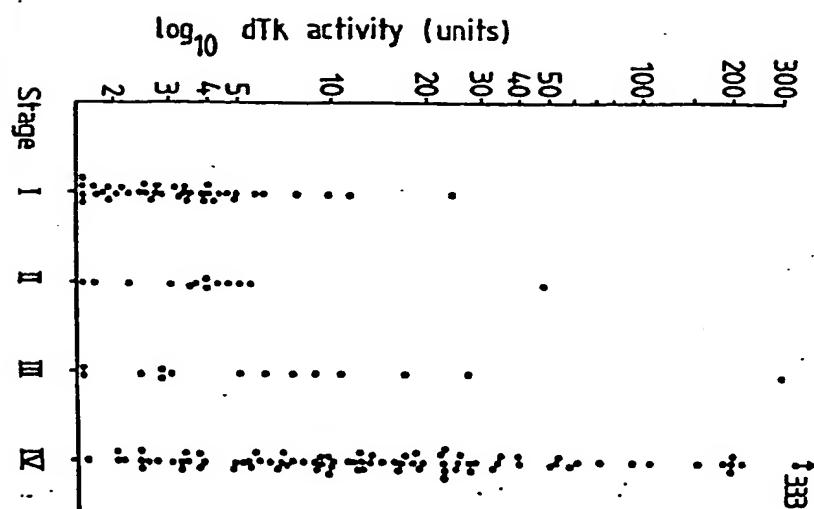


FIG. 9a

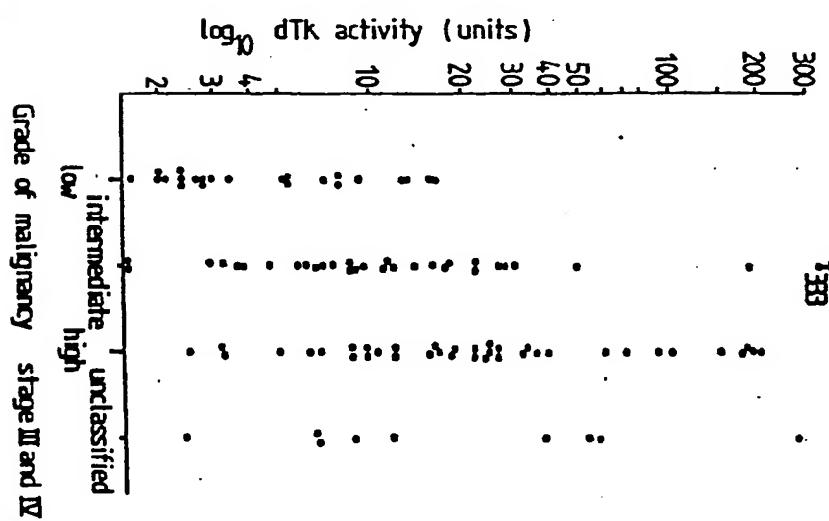


FIG. 9b

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FIG. 10a

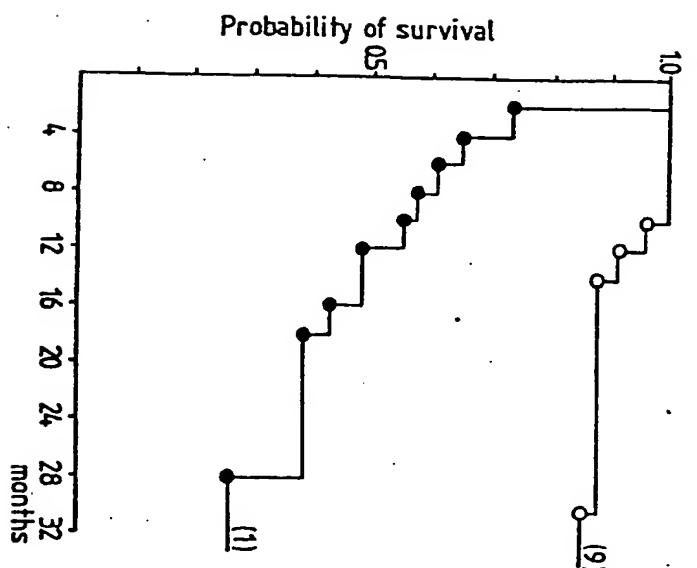
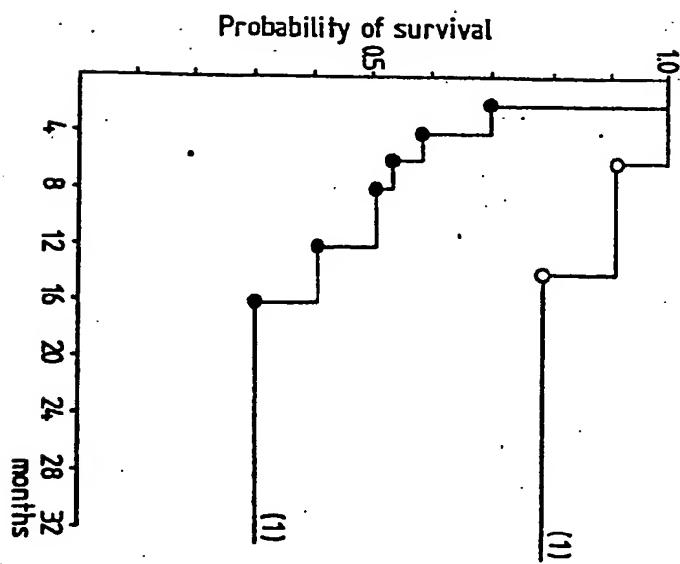


FIG. 10b



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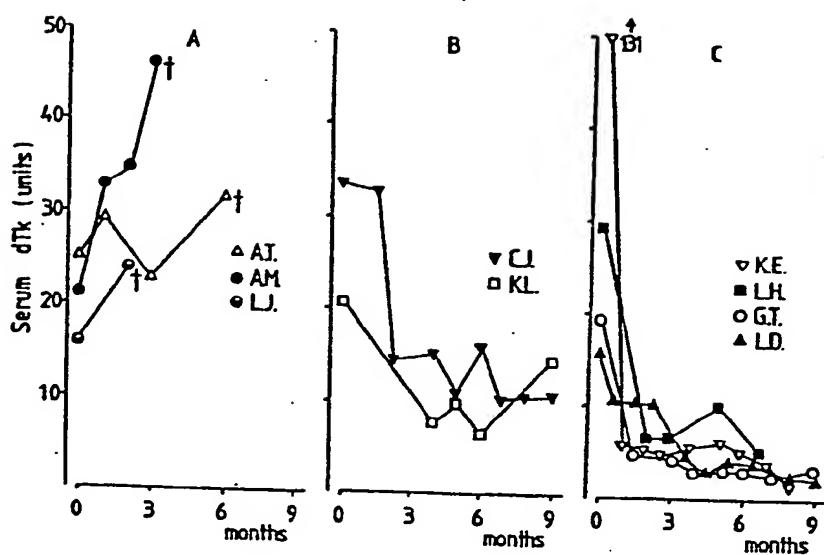


FIG. 11

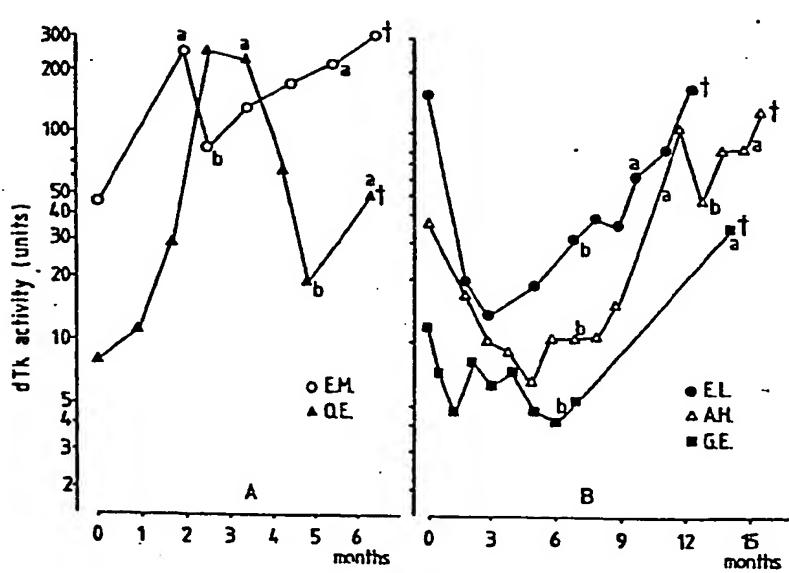


FIG. 12



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(54) A method of determining dTk isoenzyme activity and the use thereof.

(57) A method of determining dTk isoenzyme levels of a human or animal body fluid or cell sample is disclosed, which comprises the steps of reacting said sample with a substrate for said isoenzyme in the presence of a phosphate donor and a buffer system, and measuring the amount of phosphorylated product formed, said amount being proportional to said isoenzyme level. The method is characterized by using in combination

a) a buffer system at pH ranging from 5 to 9 and being present in a concentration of no more than 250 mM,  
b) a substrate consisting of 2'-deoxy-5-halouridin, part of which has a radio-labelled 5-halogen, and being present in a concentration ranging from  $2 \times 10^{-9}$  -  $5 \times 10^{-6}$  M, and  
c) the phosphate donor being present in a concentration not exceeding 20 mM.

The sensitivity of the method permits measurement of minute amounts of dTk, and the method can be used for e.g. diagnosis, prognostics and monitoring of diseases involving altered ATP mediated dTk activity, such as cancers, tumours, and certain virus infections, or altered CTP mediated dTk activity, such as HSV type 1, type 2, and VZV infections.

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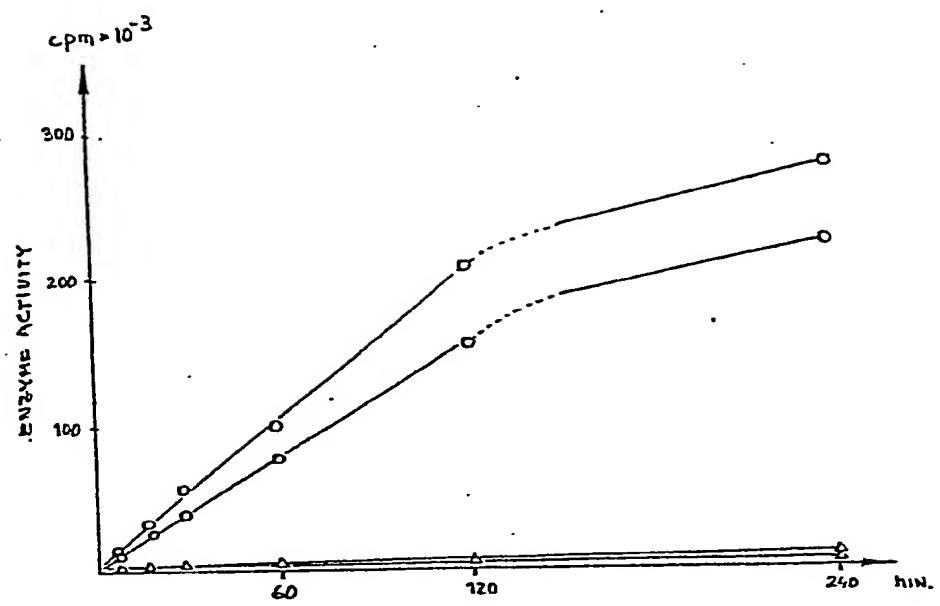


FIG 1A



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## EUROPEAN SEARCH REPORT

**0094923**  
Application number

EP 83 85 0122

DOCUMENTS CONSIDERED TO BE RELEVANT			CLASSIFICATION OF THE APPLICATION (Int. Cl. 5)
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	
D, Y	INFECTION AND IMMUNITY, vol. 29, no. 2, August 1980; J.S. GRONOWITZ "Optimized Assay for Thymidine Kinase and Its Application to the Detection of Antibodies Against Herpes Simplex Virus Type 1- and 2-Induced Thymidine Kinase", pages 425-434	1-7	C 12 Q 1/48 C 12 Q 1/50
A	idem	8	
P, Y	--- CHEMICAL ABSTRACTS, vol. 96, no. 25, 21st June 1982, Columbus, Ohio, USA; J.S. GRONOWITZ et al. "Human serum antibodies to varicella-zoster virus thymidine kinase", pages 554,555, abstract no. 215710z & Infection and immunity, vol. 36, no. 1, 1982; pages 30-37 (Cat. D)	1-7	TECHNICAL FIELDS SEARCHED (Int. Cl. 5) C 12 Q 1/00
D, A	--- JOURNAL OF MEDICAL VIROLOGY, vol. 8, 1981; J.S. GRONOWITZ et al. "Occurrence of Antibodies Against Herpes Simplex Virus Thymidine Kinase in Human Sera", Pages 177-186	6-8	
A	--- EP-A-0 042 482 (SLOAN-KETTERING INSTITUTE FOR CANCER RESEARCH) * Page II; claims 1-9 *	8	
The present search report has been drawn up for all claims			
Place of search <b>BERLIN</b>	Date of completion of the search <b>19-03-1985</b>	Examiner <b>GREEN C.H.</b>	
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